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# **Abstract**

## **Assessment of viral contamination with microbial markers in water environment**

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Noroviruses outbreaks have been continuously reported worldwide by eating noroviruses-contaminated shellfish. Particularly, the upstream of shellfish growing area is found to be sources of fecal contamination containing human pathogens such as noroviruses. In order to monitor microbial water quality, coliphages have been used as indicator of human viral contamination. This study aims to evaluate water quality

by studying coliphages for preventing public health risks associated with shellfish consumption in a shellfish growing area in South Korea. The coliphages were isolated from water samples, enriched, and then purified for getting single phage. During six sampling times, the mean levels of male-specific and somatic coliphages were  $1.34 \times 10^1$  PFU/L and  $2.93 \times 10^0$  PFU/L in surface water, whereas those coliphages in seawater samples were rarely detected. Also, both coliphages levels were highest in September sampling period. In addition, male-specific coliphages showed highly correlated with noroviruses ( $r^2 = 0.418$ ;  $P < 0.01$ ). Based on Geographic Information System analysis, hot spot of fecal and noroviruses contamination in the study area was located near the residential areas. Also, the levels of both coliphages were positively correlated with several environmental parameters, such as humidity, rainfall and temperature ( $P < 0.05$ ). It is important to have comprehensive understanding of coliphages to predict the behavior of enteric viruses in shellfish growing areas to minimize public health risks associated with shellfish consumption.

Key words: Shellfish growing area, Coliphages, Noroviruses  
Geographic Information System analysis, Environmental parameters

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# **I. Introduction**

In recent years, viral contamination from human and animal wastes has been a leading cause of waterborne diseases in worldwide (1-3). The discharge of point and nonpoint or treated and untreated wastes into water environments are a well-known sources of the fecal contamination (4, 5). Due to these fecal contamination, many coastal bays are exposed to high levels of bacteria and viruses from various fecal sources, and this is why shellfish harvest area is limited (6). Although fecal bacteria or viruses are not pathogenic to human, their existence in shellfish surface or growing areas may indicate fecal contamination including potential human pathogen and zoonotic pathogen (6, 7). Human pathogens, especially enteric viruses transmitted via fecal-oral route, can cause water and food-related infectious disease including severe viral gastroenteritis. In human fecal samples, more than 140 human enteric viruses have been identified, and these viruses can cause human viral gastroenteritis (4). Especially, noroviruses were highly infectious and associated with severe disease in humans (4). The epidemiological evidence revealed that gastroenteritis caused by human noroviruses were associated with the shellfish consumption (8).

For preventing exposure of fecal source including pathogens, water environments act as primary sources should be controlled and managed, and surveillance of fecal contamination should be conducted to monitor water quality in shellfish growing areas. Microbial source tracking (MST) tool is not a standard environmental monitoring methods, but it has been extensively applied and investigated in nations worldwide (9-13). For testing water quality, microbial indicators such as *Escherichia coli* and enterococci have been widely used as standards worldwide (14-16). However, many studies have described that fecal indicator bacteria (FIB) levels have shown a lack of correlation with the concentration of enteric viruses (17). Instead of FIB, coliphages that infect coliform bacteria, somatic and male-specific coliphages have been suggested for alternative indicators of human viral contamination for monitoring of microbial water quality (4, 18, 19). Even though molecular tests have been developed to directly detect enteric viruses in water environments, the monitoring of various enteric viruses is not practical because it is time-consuming and expensive (4, 20). However, coliphages as surrogate of enteric viruses have been extensively used as fecal markers because it is practical than enteric viruses, and their characteristics are more similar to enteric viruses such as morphology, size, physiology, and persistence in water environment (4, 20, 21).

In surface water, the concentration of fecal indicators could be affected from many factors including point and nonpoint sources (22). Previous study has shown that the fecal contamination of water environments is influenced by the areas near the surface water (23). In recent years, the increasing population size and its subsequent change have had a significant effect on the land use pattern. To improve water quality from the contamination sources, land use should be controlled and monitored, and the site-specific mitigation strategies is needed to reduce the exposure of fecal contaminant to water environment (24). In addition, as many studies argued that water quality can be influenced by not only land use but environmental factors such as rainfall (25, 26), suggesting they should be studied together.

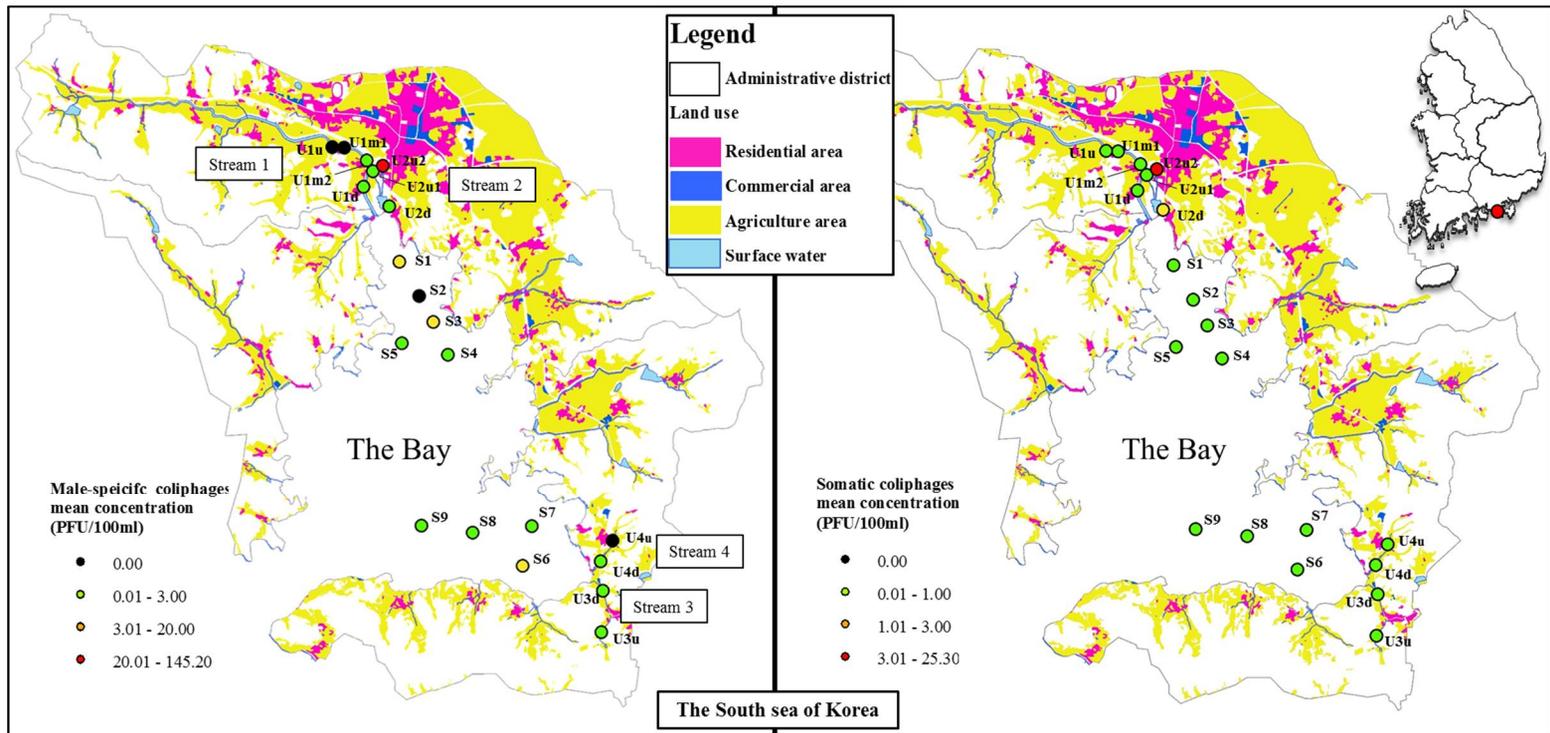
Because of importance of water environments, a number of countries have carried out investigation of fecal contamination in shellfish growing areas (6, 21, 27, 28). Several studies were conducted to investigate fecal contamination in shellfish growing area, but most of seawater investigations have been based on bacterial criteria (29, 30). Several studies have argued that current controls may be inadequate to reduce norovirus gastroenteritis because viruses are more persistence in the environment than fecal bacterial indicator and eliminated slowly

from shellfish (30-32). To prevent virus contamination such as norovirus in water environment, coliphages are necessary to evaluate the viral and fecal contamination in shellfish growing area. The aims of this study were i) to examine the distribution of the two types of coliphages in the surface water and seawater of actively producing shellfish area to minimize the public health risks from the contamination of enteric viruses. In addition, this study was applied ii) to evaluate the potential relationship between coliphages and environmental parameters, iii) to investigate possible sources of fecal contamination from land use by analyzing GIS, and iv) to identify relationship between noroviruses and microbial indicators

## **II. Materials and Methods**

### **1. Sampling sites and collection of water samples**

The South Sea, well known place for actively producing shellfish, was selected as investigation site. According to previous study, Gyeongnam region, located in the South Sea of Korea, produced the largest volume of oysters in Korea (29). Our study area, part of Gyeongnam region, has 1,989 ha of fishery and 11,590 tons of shellfish culturing area. Nine seawater sampling sites and eleven sites were chosen from its upstream surface water, respectively (Fig. 1). The surface water has four stream lines and each of stream lines has 4, 3, 2 and 2 sampling sites, respectively. In seawater sampling sites, the S1 through S5 was located near the stream 1 and 2, and the S6 through S9 is placed near stream 3 and 4. Upper case U or S is for surface water and seawater, respectively, and upstream of surface water is coding as lower case 'u', middle stream as 'm' and downstream as 'd'. The water samples were collected six times in March, May, July, September, and December, 2015 and January, 2016. Total 120 water samples were collected from 20 sampling sites.



**Figure 1.** The mapping of sampling sites at shellfish growing areas in South Korea and land use analysis around sampling sites by GIS. The left figure is described for male-specific coliphages and the right figure is for somatic coliphages. The upper case U is for surface water and S for seawater. Sites are coded based on their respective stream number.

## 2. Environmental data

Several environmental parameters such as average temperature, wind speed, humidity, precipitation level on the sampling day (prep-0), the total precipitation for 14 days (prep-14) before sampling day, were obtained from Korea Meteorological Administration (33), and water temperature, pH, conductivity, salinity and turbidity were measured *in situ* using YSI multi parameter instrument (Professional Plus; Yellow Springs Instruments, Yellow Springs, OH, USA).

### 3. Sample concentration

Method 1615 provided by USEPA was applied to concentrate virus particles in environmental water samples (34). Briefly, each NanoCeram<sup>®</sup> cartridge filters (Argonide Corporation, Sanford, FL, USA) filtrated by 100 L of environmental waters were stored at 4 °C and processed within 72 h. . In elution process, cartridge filters were incubated with 3.0% beef extract (pH9.5; BD Bioscience, San Jose, CA, USA) solution containing 0.1 M glycine (Duchefa, St. Louis, MO, USA) for 5 min by three times. pH of the eluate samples were adjusted to 3.5 with 1 M HCl and stirred for 30 min in room temperature. After centrifugation of  $2,500 \times g$  at 4 °C for 15 min, the pellet was completely suspended by 21 ml of 0.15 M sodium phosphate ( $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ , pH 9.0–9.5). Supernatants were collected by secondary centrifugation of  $10,000 \times g$  at 4 °C for 10 min. After pH of the supernatants were adjusted to 7.0–7.5 with 1 M HCl, each samples was finally filtrated using 0.22- $\mu\text{m}$  pore sized syringe filter, and stored at -80 °C until analysis. The average volume of the final eluate was 21.1 ml (Min: 12.8 ml – Max: 23 ml).

#### **4. Analysis of coliphages**

The EPA method 1602, single agar layer (SAL) method, was used to isolate coliphages from seawater and surface water samples (35). The concentrated sample (0.5 mL) and the host (0.3 mL), F<sub>amp</sub> host (ATCC 700891) for male-specific coliphages or CN13 host (ATCC 700609) for somatic coliphages were added with 29 mL of tryptic soy agar (Becton, Dickinson and Company, Sparks, MD, USA). After solidification, agar plates were incubated at 37°C for 16–18 h to yield plaques. Plaques were counted and virus titers recorded as numbers of plaque forming unit (PFU)/100 mL. For enrichment, a single plaque, isolated from the plate using a sterile pipette tip, was suspended in 5 mL of tryptic soy broth with adding 100 µL of host, and was cultivated in shaking incubator with 150 rpm at 37°C for 16–18 h. After enrichment, 5 mL of chloroform was added and centrifuged at 5,000 × g for 20 min for purification, and then, a single coliphage was finally isolated from the supernatant. Isolated coliphages are stored at -80°C until further analysis. Coliphages MS2 (ATCC 15597-B1) and PhiX174 (ATCC 13706-B1) was used as positive controls for male-specific coliphages and somatic coliphages respectively. Autoclaved phosphate saline buffer was used as a negative control.

## **5. RNase sensitivity assay to differentiate DNA and RNA coliphages**

RNase sensitivity assay was performed to characterize male-specific coliphages isolated. The host, mixing with 0.8% TSA agar and RNase or without RNase (100 ng/mL; Sigma-Aldrich, St. Louis, MO, USA), were poured into petri dish. Isolated coliphages and 10-fold dilution of those coliphages were spotted onto both with or without RNase petri dish. The petri dishes were incubated at 37°C for 16–18 h. If coliphages were only visible in RNase-negative petri dishes, those coliphages were considered RNA coliphages. When coliphages were recovered in both RNase positive and negative petri dishes, those coliphages were considered DNA coliphages.

## 6. Quantification of noroviruses

The duplex real-time RT-qPCR assays were performed in 25  $\mu\text{L}$  reaction mixtures containing 5  $\mu\text{L}$  of samples, 12  $\mu\text{L}$  of 2 $\times$  RT-PCR buffer, 0.5  $\mu\text{L}$  of 25 $\times$  enzyme mix, 1.5  $\mu\text{L}$  of detection enhancer using the Agpath-ID One-Step RT-PCR (Thermo Fisher Scientific Inc, Waltham, MA), 1  $\mu\text{L}$  (10 pmol/  $\mu\text{L}$ ) of COG1F (GI forward primer) and COG1R (GI reverse primer), 0.5  $\mu\text{L}$  (10 pmol/  $\mu\text{L}$ ) of RING1(a)-TP (GI probe), 1  $\mu\text{L}$  (10 pmol/  $\mu\text{L}$ ) of BPO 13 and 13N (GII forward primers) and 14 (GII reverse primer), and 0.5  $\mu\text{L}$  (10 pmol/  $\mu\text{L}$ ) of BPO18 GII probe (36). The sequences of primers and probes for GI and GII assay was prescribed in Table 1. The detection of noroviruses in water samples was performed in the C1000 Thermal Cycler CFX96 Real-time PCR system (Bio-Rad, Hercules, CA). For detection of noroviruses, the real-time PCR was performed that reverse transcription 30 min at 45°C, initial denaturation step at 95°C for 10 min, followed by 45 cycles of denaturation for 10 s at 95°C, and 60 s of annealing at 56°C. Viral copy number was quantified using dilutions of Norovirus RNA Positive Control (AccuPower® Norovirus Real-Time RT-PCR Kit; Bioneer, Daejeon, Republic of Korea). All samples were run in triplicates and each assay included a triplicate of no template controls.

**Table 1.** Oligonucleotide sequence for quantification of noroviruses.

Type	Primer & Probe	Sequence (5' → 3' )	<sup>a</sup> Location
<b>GI</b>	COG1F	CGYTGGATGCGNTTYCATGA	5291
	COG1R	CTTAGACGCCATCATCATTYAC	5375
	RING1(a)-TP	FAM-AGATYGCGATCYCCTGTCCA-TAMRA	5340
<b>GII</b>	BPO-13	AICCIATGTTYAGITGGATGAG	5007
	BPO-13N	AGTCAATGTTTAGGTGGATGAG	
	BPO-14	TCGACGCCATCTTCATTCACA	5101
	BPO-18	VIC-CACRTGGGAGGGCGATCGCAATC-TAMRA	5044

<sup>a</sup>GI primer sequences correspond to position in Norwalk/68 virus (accession no. M87661);

GII primer sequences correspond to position in Lordsdale virus (accession no. X86557)

## **7. Analysis of coliforms**

Coliforms (total and fecal coliforms) were measured using the 5-tube most probable number (MPN) method (37). Each diluted water sample was first inoculated into lauryl tryptose broth and incubated at 35°C for 48 h. Gas-generated cultures were transferred into brilliant green bile broth (Oxoid, Hampshire, UK) and incubated at 35°C for 48 h and EC broth (BD, Franklin Lakes, NJ, USA) and incubated at 44.5°C for 24 h to test for total and fecal coliforms, respectively. After incubation, positive tubes were counted and assessed using MPN tables.

## **8. Analysis of GIS data**

Analysis of all GIS data were performed by ArcGIS (ESRI, 10.2.2, CA, USA). GIS data was based on watershed of near the sampling site and was used to investigate the land use of the near sampling sites. The administrative divisions, land use maps were obtained from the Korea water resources management information system (WAMIS) (38).

## 9. Statistical analysis

The data was statistical analyzed with the IBM® SPSS® (Release ver. 18.0.0; SPSS Inc., Chicago, IL, USA), bar graphs were edited by Graphpad prism 5 (GraphPad software, Inc., San Diego, CA, USA), and the heatmap analysis of correlation coefficients were obtained by R statistics for windows ver. 3.3.2 (Lucent Tech, USA). Nonparametric Kruskal-Wallis test was conducted for comparing three or more groups, and the alpha 0.05 subcommand was used to further analyze the data by Dunn's multiple comparison test. Mann-Whitney test was used to test difference between the two groups. The heatmap analysis showed spearman correlation coefficients between coliphages and environmental parameters. Also norovirus and indicators including coliform and coliphages were performed by spearman correlation. All statistical analysis was evaluated at the 95% confidence level.

### **III. Results**

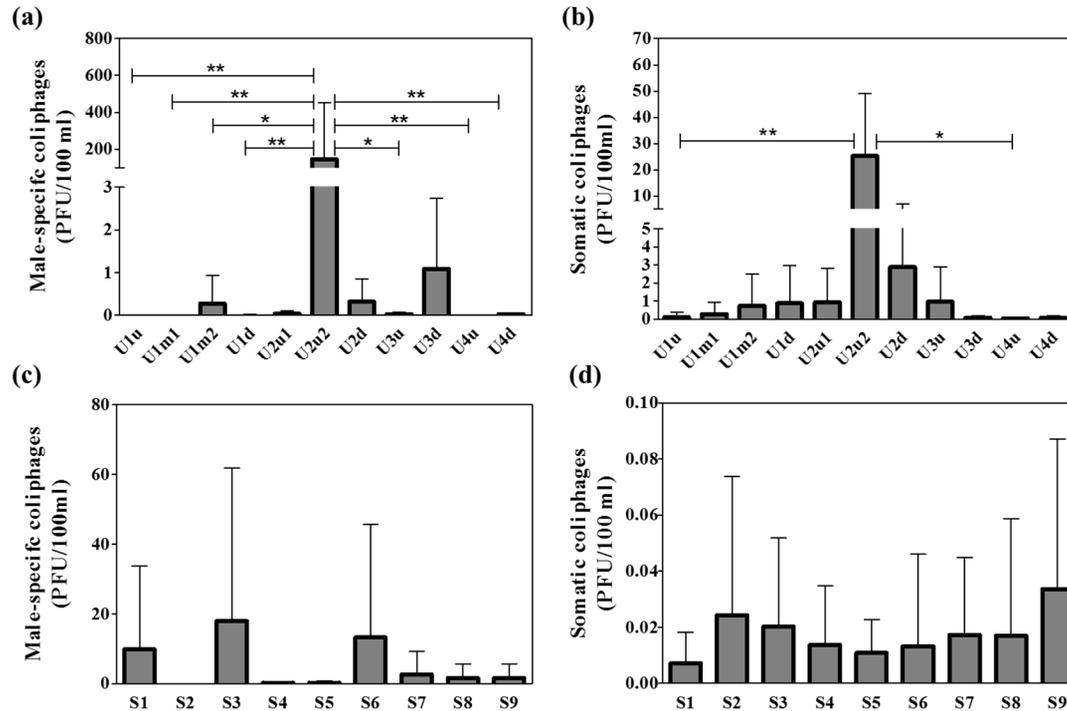
#### **1. Distribution coliphages in surface and seawater samples**

The results of male-specific and somatic coliphages in surface water and seawater samples during the sampling period are presented in Fig. 2 and Table 2. The mean concentrations of male-specific and somatic coliphages for all 66 surface water samples were  $1.34 \times 10^1$  PFU/100 mL (0 to  $7.73 \times 10^2$  PFU/100 mL) and  $2.93 \times 10^0$  PFU/100 mL (0 to  $7.05 \times 10^1$  PFU/100 mL), respectively (Table 2). The male-specific coliphages were positive in 19 out of 66 (29%) samples, and somatic coliphages were observed with 38 positive samples out of 66 samples (58%). However, in seawater samples, there were showed lower detection frequency for both coliphages. The mean concentrations of male-specific coliphages and somatic coliphages in seawater samples were  $5.25 \times 10^0$  PFU/100 mL (0 to  $1.07 \times 10^2$  PFU/100 mL) and  $2.00 \times 10^{-2}$  PFU/100 mL (0 to  $1.24 \times 10^{-1}$  PFU/100 mL), respectively. The male-specific coliphages were observed in 8 out of 54 samples (15%) and somatic coliphages were showed in 17 out of 54 samples (31%).

The concentration of male-specific coliphages in U2u2 site was higher than those in other surface water sampling sites ( $1.45 \times 10^2 \pm 3.08 \times 10^2$  PFU/100 mL; Fig. 2a), and the detection frequency of male-specific coliphages in U2u2 was 6 out of 6 sampling times (100%; Table 3). However, the other sampling sites were detected less than 2 PFU/100 mL (Fig. 2). The concentration of male-specific coliphages was significantly different (Kruskal-Wallis test;  $P = 0.0001$ ) between U2u2 and U1u, U1m1, U1m2, U1d, U3u, U4u, and U4d (Dunn's multiple comparison test;  $P < 0.05$ ).

Somatic coliphages showed a similar trend with male-specific coliphages (Fig. 2b). For somatic coliphages, U2u2 site also showed the highest concentration ( $2.52 \times 10^1 \pm 2.38 \times 10^1$  PFU/100 mL), and the detection frequency of somatic coliphages in U2u2 site was also 6 out of 6 (100%). The second highest mean concentration of somatic coliphages were in U2d ( $2.89 \times 10^0 \pm 4.12 \times 10^0$  PFU/100 mL), and the detection frequency of somatic coliphages was 5 out of 6 (83%). The distribution of somatic coliphages was statistically different among the sampling sites (Kruskal-Wallis test;  $P = 0.0139$ ), especially between U2u2 and U1u, and U2u2 and U4u (Dunn's multiple comparison test;  $P < 0.05$ ).

In seawater sampling sites, the mean concentration of male-specific coliphages were the highest in S3 ( $1.79 \times 10^1 \pm 4.39 \times 10^1$  PFU/100 mL; Fig 2c), which is located near Stream 1 and Stream 2 (Fig. 1), but they were observed in only 1 out of 6 (17%) samples (Table 3). For somatic coliphages, the Site S9, which is located near Stream 3 and Steam 4 (Fig. 2d), showed the highest level ( $3.37 \times 10^{-2} \pm 5.35 \times 10^{-2}$ PFU/100 mL), and 2 out of 6 (33%) samples were positive.



**Figure 2.** The bar graphs of average concentration and standard deviation for male-specific coliphages (a) and somatic coliphages (b) by sampling sites from surface water, and for male-specific coliphages (c) and somatic coliphages (d) by sampling sites from seawater. Statistical significance values: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$

**Table 2.** Summary of the concentration and detection frequency of coliphages in surface and seawater samples.

Indicators	Surface water (n=66)			Seawater (n=54)		
	Mean (Median)	Range	No. positive samples/no. total samples (%)	Mean (Median)	Range	No. positive samples/no. total samples (%)
<b>Male-specific coliphage (PFU/100 mL)</b>	$1.34 \times 10^1$ (0.0)	0.00 ~ $7.73 \times 10^2$	19/66 (29%)	$5.25 \times 10^0$ (0.0)	0.00 ~ $1.07 \times 10^2$	8/54 (15%)
<b>Somatic coliphage (PFU/100 mL)</b>	$2.93 \times 10^0$ (0.0)	0.00 ~ $7.05 \times 10^1$	38/66 (58%)	$2.00 \times 10^{-2}$ (0.0)	0.00 ~ $1.24 \times 10^{-1}$	17/54 (31%)

**Table 3.** Detection frequency of coliphages in surface water sampling sites.

Water types	Indicator	Sampling sites										
		U1u	U1m1	U1m2	U1d	U2u1	U2u2	U2d	U3u	U3d	U4u	U4d
Surface water	Male-specific coliphages	0%	0%	17%	17%	33%	100%	50%	17%	67%	0%	17%
	Somatic coliphages	17%	50%	50%	50%	50%	100%	83%	50%	67%	50%	67%
Seawater		S1	S2	S3	S4	S5	S6	S7	S8	S9		
	Male-specific coliphages	17%	0%	17%	17%	17%	17%	17%	17%	17%		
	Somatic coliphages	33%	33%	33%	33%	50%	17%	33%	17%	33%		

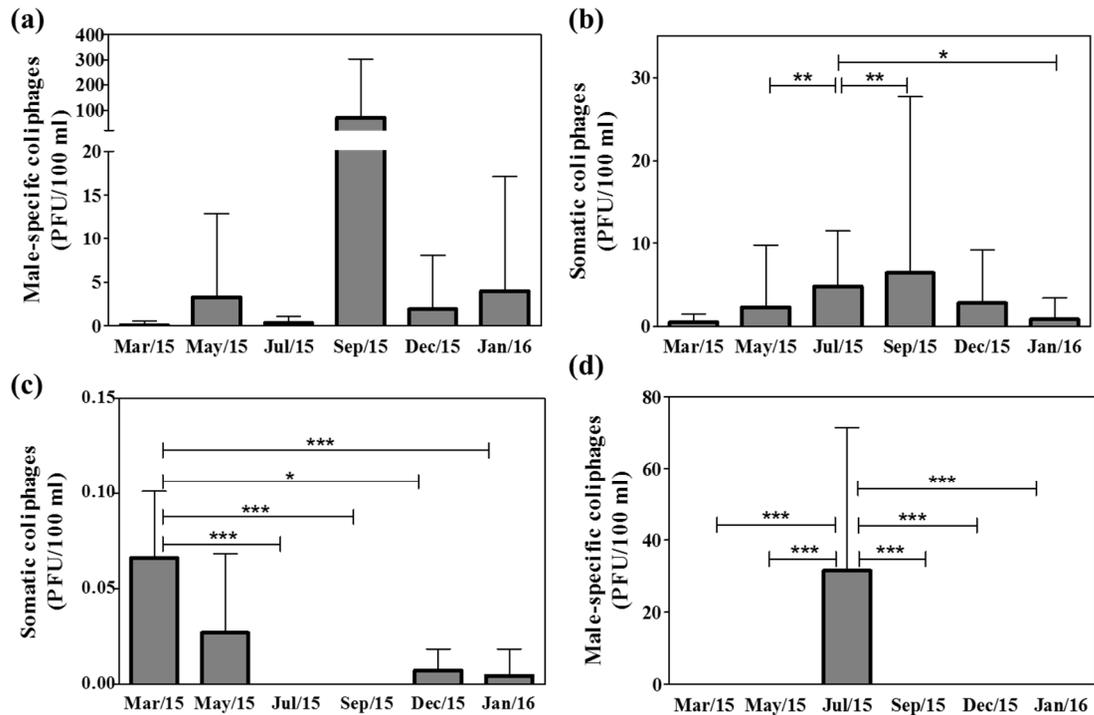
## **2. Seasonal distribution of coliphages during sampling periods**

Fig. 3 and Table 4 showed the distribution and detection frequency of coliphages in surface water and seawater samples at every sampling times. For surface water samples, the highest concentration of male-specific coliphages was shown in September ( $7.04 \times 10^1 \pm 2.33 \times 10^2$  PFU/100 mL; Fig. 3a) and their detection frequency was 27% (3 out of 11 samples; Table 4). On the other hand, both the mean concentration ( $1.27 \times 10^{-1} \pm 4.42 \times 10^{-1}$  PFU/100 mL) and the detection frequency of coliphages (1 out of 11; 9%) were lower in March. The distribution of somatic coliphages were also higher in September ( $6.42 \times 10^0 \pm 2.12 \times 10^1$  PFU/100mL; Fig 3b) and their detection frequency was 36% (4 out of 11 samples), whereas the detection frequency was higher in March and July (10 out 11 samples; 91%; Table 4).

Statistical analysis showed the distribution of somatic coliphages was significantly different among each sampling time (Kruskal-Wallis test,  $P < 0.001$ ); the distribution of somatic coliphages was significantly different between May and July, between July and September, and between July and January samples (Dunn's multiple

comparison test;  $P < 0.05$ ). On the other hand, there was no significant difference of the distribution of male-specific coliphages among sampling sites (Kruskal-Wallis test,  $P > 0.05$ ).

For seawater samples, only July samples were positive for male-specific coliphages ( $3.15 \times 10^1 \pm 4.01 \times 10^1$  PFU/100 mL; Fig. 3c), and 8 out of 9 samples were positive (89%; Table 4). The distribution of male-specific coliphages was statistically different (Kruskal-Wallis test,  $P < 0.0001$ ) between July and other sampling months (Dunn's multiple comparison test;  $P < 0.001$ ). Somatic coliphages were detected from the samples taken from March, May, December and January samples, but their mean concentration was very low ( $< 1.00 \times 10^{-1}$  PFU/100 mL; Fig. 3d). Statistical analysis showed the distribution of somatic coliphages was significant different (Kruskal-Wallis test,  $P < 0.0001$ ) between March and July, September, December and January samples (Dunn's multiple comparison test;  $P < 0.05$ ).



**Figure 3.** The bar graphs of average concentration and standard deviation for male-specific coliphages (a) and somatic coliphages (b) during sampling periods from surface water, and for male-specific coliphages (c) and somatic coliphages (d) during sampling periods from seawater. Statistical significance values: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

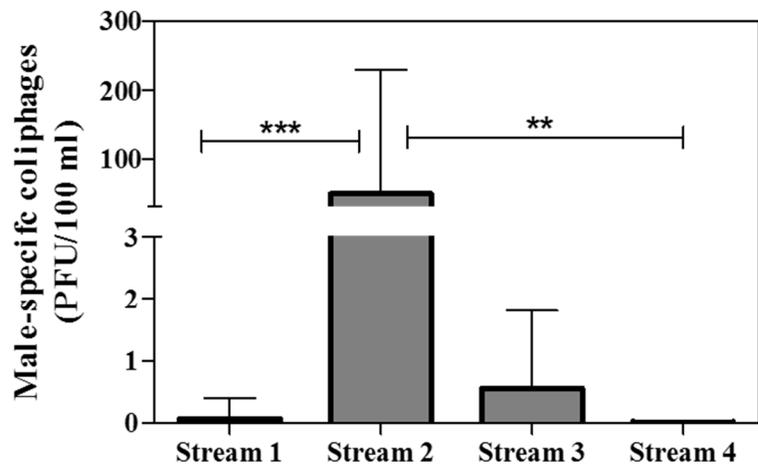
**Table 4.** Detection frequency of coliphages over sampling periods.

<b>Water type</b>	<b>Indicator</b>	<b>March 2015</b>	<b>May 2015</b>	<b>July 2015</b>	<b>September 2015</b>	<b>December 2015</b>	<b>January 2016</b>
<b>Surface water</b>	<b>Male-specific coliphages</b>	9%	18%	36%	27%	36%	45%
	<b>Somatic coliphages</b>	91%	27%	91%	36%	64%	36%
<b>Seawater</b>	<b>Male-specific coliphages</b>	0%	0%	89%	0%	0%	0%
	<b>Somatic coliphages</b>	100%	44%	0%	0%	33%	11%

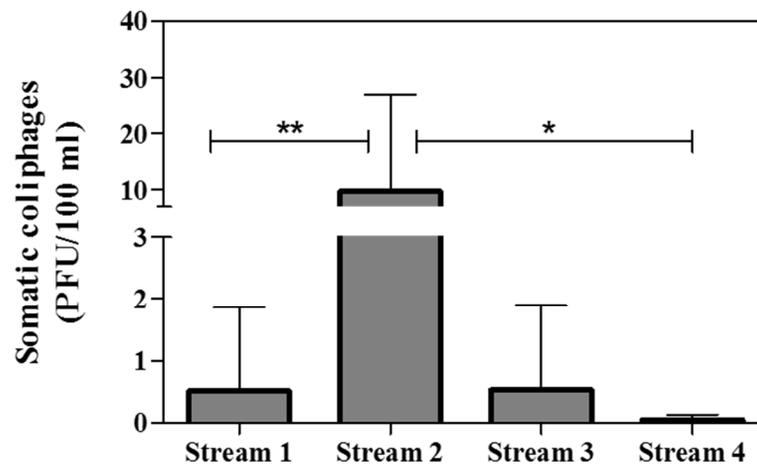
### **3. Comparison of levels of coliphages among four stream lines in surface water**

In surface water, the four stream lines showed different distribution of coliphages (Fig. 4). Stream 2 showed the highest contamination of male-specific and somatic coliphages ( $4.85 \times 10^1 \pm 1.81 \times 10^2$  PFU/100 mL and  $9.70 \times 10^0 \pm 1.73 \times 10^1$  PFU/100 mL). Stream 2 showed statistical difference with the other streams (Kruskal-Wallis test,  $P < 0.001$ ). Male-specific coliphages were significantly different between Stream 1 and Stream, and Stream 2 and Stream 4 (Dunn's multiple comparison test;  $P < 0.01$ ; Fig. 4a). Somatic coliphages also showed similar trend as male-specific coliphages. The distribution of somatic coliphages showed a significant difference among 4 streams (Kruskal-Wallis test,  $P < 0.01$ ). Stream 1 and Stream 2, and Stream 2 and Stream 4 (Dunn's multiple comparison test;  $P < 0.05$ ; Fig. 4b) were significantly different.

(a)



(b)



**Figure 4.** The bar graphs of average concentration and standard deviation of male-specific (a) and somatic coliphages (b) for four streams in surface water. Statistical significance values: \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ .

#### **4. The Environmental parameters**

The results of environmental parameters at both surface and seawater are presented in Table 5. The average temperature was higher in July and September in surface (22.7 °C and 21.1 °C, respectively) and seawater (23.9 °C and 21.2 °C, respectively). Also, the accumulative of precipitation for 14 days before sampling day was highest in July period for surface and seawater (141.6 mm and 141.7 mm, respectively). The average humidity was highest in July for both surface and seawater samples (94% and 94.1%, respectively) whereas the lowest in January for both surface and seawater samples (55.5% and 49.4%, respectively). The average water temperature was higher in July and September for surface water (25.2°C and 22.1°C, respectively) and for seawater (23.9°C and 24.7°C, respectively).

Sampling Time (Month/year)	Average Temperature (°C)		Average Wind Speed (m/s)		Average Humidity (%)		<sup>a</sup> Prep-0 (mm)		<sup>b</sup> Prep-14 (mm)	
	Surface	Sea	Surface	Sea	Surface	Sea	Surface	Sea	Surface	Sea
Mar/2015	13.90	13.0	2.0	1.7	79.9	72.1	0.0	0.0	70.0	70.00
May/2015	16.9	17.0	4.3	1.9	80.8	71.9	73.7	0.0	119.3	119.30
Jul/2015	22.7	23.9	2.7	1.9	94.0	94.1	0.0	3.1	141.6	141.70
Sep/2015	21.1	21.2	2.7	2.1	68.8	66.3	0.5	0.0	29.0	24.20
Dec/2015	10.7	9.9	2.5	2.7	83.5	72.5	2.9	0.6	40.0	40.00
Jan/2016	2.2	0.8	4.8	1.7	55.5	49.4	0.0	0.0	8.1	5.90

Sampling Time	Water Temperature (°C)		pH		Turbidity (NTU)		Salinity (psu)	
	Surface	Sea	Surface	Sea	Surface	Sea	Surface	Sea
Mar/2015	19.3	12.4	7.8	7.4	6.1	1.2	1.5	33.5
May/2015	19.6	19.3	7.5	8.2	5.7	1.1	1.5	32.6
Jul/2015	25.2	23.9	7.2	8.2	11.1	2.4	1.6	33.1
Sep/2015	22.1	24.7	7.3	8.1	6.8	2.4	0.2	29.4
Dec/2015	13.0	10.9	7.4	8.1	5.8	2.8	2.1	32.8
Jan/2016	8.1	3.2	6.7	7.0	6.7	2.0	2.2	33.1

**Table 5.** Summary of the average meteorological information and physiochemical by sampling periods.

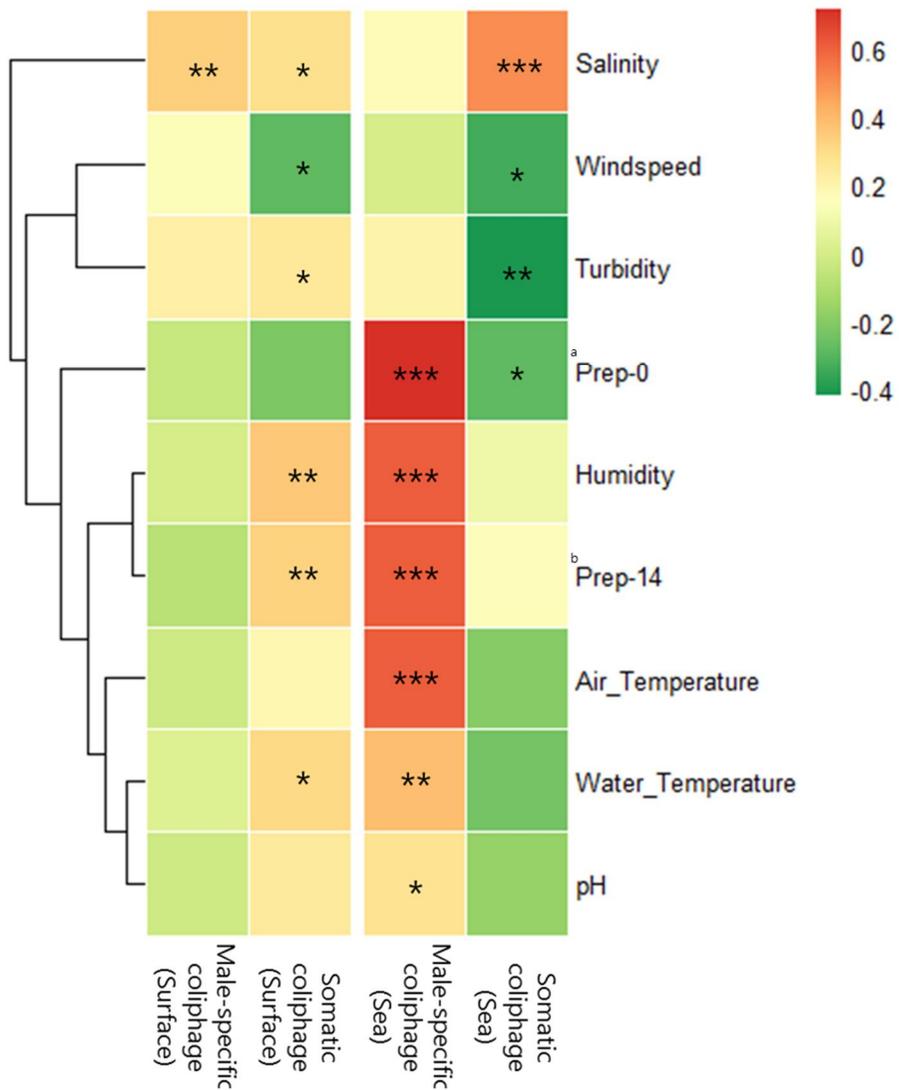
<sup>a</sup> The accumulative precipitation at sampling day.

<sup>b</sup> The accumulative 14-day precipitation before sampling day.

## **5. The relationship between environmental parameters and coliphages**

The heatmap analysis showed spearman correlation between environmental parameters and male-specific and somatic coliphages levels in surface and seawater (Fig. 5). From surface water samples, male-specific coliphages were positively correlated with salinity ( $r^2 = 0.335$ ;  $P < 0.05$ ). Somatic coliphages were correlated with water temperature ( $r^2 = 0.308$ ), salinity ( $r^2 = 0.283$ ), turbidity ( $r^2 = 0.250$ ), humidity ( $r^2 = 0.357$ ) and prep-14 ( $r^2 = 0.328$ ), whereas they were negatively correlated with average wind speed ( $r^2 = -0.286$ ;  $P < 0.05$ ).

From seawater samples, male-specific coliphages were positively correlated with prep-0 ( $r^2 = 0.719$ ), humidity ( $r^2 = 0.608$ ), prep-14 ( $r^2 = 0.608$ ) and air temperature ( $r^2 = 0.608$ ;  $P < 0.001$ ). Somatic coliphages were negatively correlated with salinity (wind speed ( $r^2 = -0.340$ ), Prep-0 ( $r^2 = -0.286$ ) and turbidity ( $r^2 = -0.419$ ;  $P < 0.05$ ) whereas positively correlated with salinity ( $r^2 = 0.502$ ;  $P < 0.001$ ).



**Figure 5.** The heatmap analysis of the spearman correlation between coliphages levels and environmental parameters in surface and seawater. <sup>a</sup>Prep-0 is the accumulative precipitation at sampling day and <sup>b</sup>prep-14 is the accumulative 14-day precipitation before sampling day. Statistical significance: \* P<0.05; \*\* P<0.01; \*\*\* P<0.001.

## **6. Characterization of potential sources of fecal contamination via land use**

Land use near the sampling sites was investigated by GIS analysis (Fig. 1). The sampling sites on Stream 1, which were located near the agriculture areas, showed low mean concentration of both coliphages (below  $1.00 \times 10^0$  PFU/100 mL). However, the stream 2, especially the U2u2 sampling site in which the concentration and the detection frequency of both coliphages were higher, was located near the concentrated residential areas. The sampling sites located on Stream 3 and Stream 4 showed the low level of coliphages, and there were few agriculture areas and residential areas near the sampling sites.

## 7. The relationship between noroviruses and indicators

The relationship between norovirus GI and GII and microbial indicators were analyzed using spearman correlation at a level of significance  $P < 0.05$ . The male-specific coliphages were highly correlated with noroviruses (GI and GII) than other indicators ( $r^2 = 0.443$ ,  $P < 0.001$ ; Table 6). Somatic coliphages also showed higher correlation coefficient with noroviruses ( $r^2 = 0.339$ ,  $P < 0.01$ ). The male-specific and somatic coliphages were more correlated with norovirus GI ( $r^2 = 0.418$ ,  $r^2 = 0.328$ , respectively,  $P < 0.01$ ) than GII ( $r^2 = 0.393$ ,  $r^2 = 0.275$ , respectively,  $P < 0.05$ ) whereas the correlation between noroviruses (GI and GII) and coliforms were lower than coliphages ( $r^2 = 0.325$  for total coliforms,  $r^2 = 0.260$  for fecal coliforms, respectively,  $P < 0.05$ ).

According to presence of norovirus, each indicator was divided by norovirus positive and negative group (Fig. 6). In noroviruses-positive samples, the mean concentrations of male-specific and somatic coliphages were higher than those of noroviruses-negative group (Mann Whitney test,  $P < 0.05$ ; Fig. 6a and 6b). However, coliforms

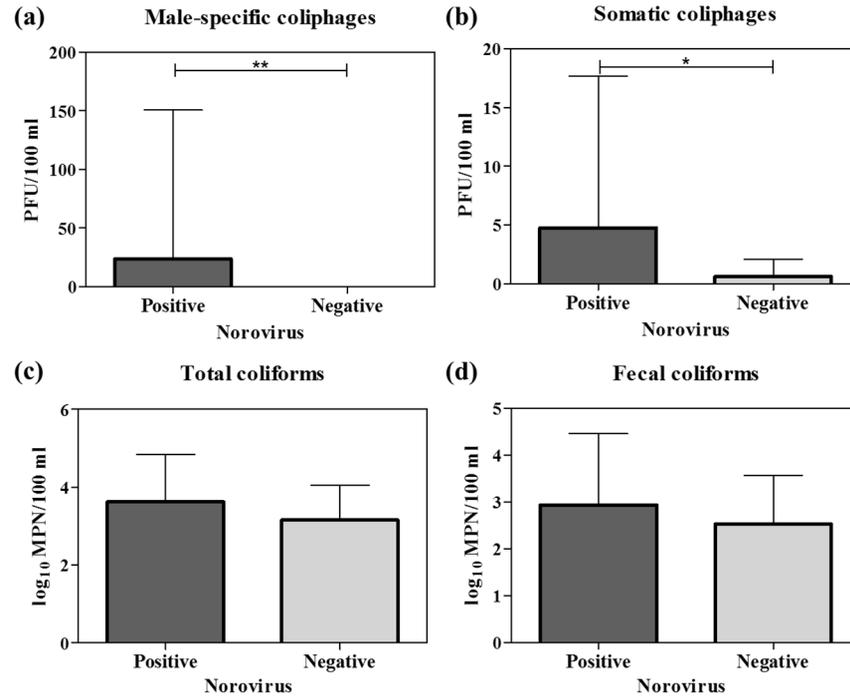
were highly detected in both noroviruses-negative and -positive groups (Fig. 6c and 6d).

The detection frequency of coliphages and noroviruses showed a similar site-specific pattern. For instance, noroviruses were highly detected in U2u2 and U3u sites than other sites (Fig. 7a), whereas coliforms were detected in all sampling sites at surface water regardless of the presence of noroviruses (Fig. 7c). On the other hand, noroviruses were prevalent in winter than summer season whereas coliphages were prevalent in late summer than winter season (Fig. 7b). However, no apparent seasonality was shown in coliforms data (Fig. 7d).

**Table 6.** Spearman correlation between noroviruses and indicators, and their coefficients.

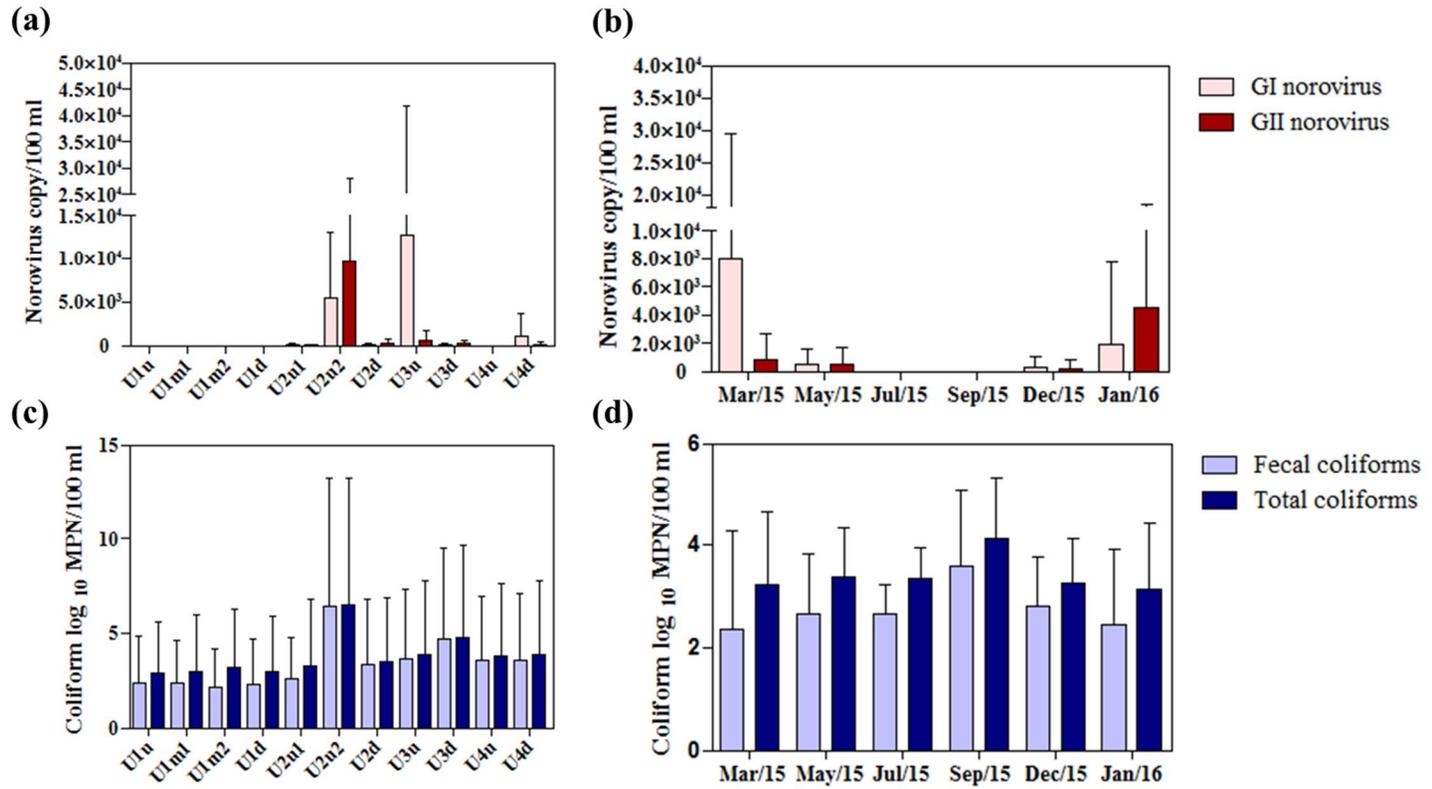
Types	Norovirus GI	Norovirus GII	Norovirus (GI+GII)	Total Coliforms	Fecal Coliforms	Male-specific Coliphages	Somatic Coliphages
Norovirus GI							
Norovirus GII	<sup>a</sup> 0.618***						
Norovirus (GI + GII)	0.833***	0.896***					
Total coliforms	0.288*	0.313*	0.325**				
Fecal coliforms	0.277*	0.257*	0.260*	0.866***			
Male-specific coliphages	0.418***	0.393**	0.443***	0.545***	0.489***		
Somatic coliphages	0.328**	0.275*	0.339**	0.354**	0.342**	0.460***	

<sup>a</sup> Statistical significance: \* P<0.05; \*\* P<0.01; \*\*\* P<0.001.



**Figure 6.** The bar graphs of average concentration and standard deviation of male-specific (a), somatic coliphages (b), Total coliforms (c), and fecal coliforms (d) according to presence of norovirus in surface water.

Statistical significance values: \*P < 0.05; \*\*P < 0.01



**Figure 7.** The bar graphs of average concentration and standard deviation of norovirus and coliforms by sampling sites (a) and (c) respectively, and by sampling period (b) and (d), respectively in surface water.

## **IV. Discussion**

Male-specific DNA and somatic coliphages were widely observed in human and nonhuman fecal sources. In previous study, male-specific DNA coliphages were highly observed in wastewater treatment plants (human sewage) and bovine waste water, whereas male-specific DNA and RNA coliphages were equally identified in swine waste (18). In addition, male-specific DNA coliphages were characterized from raw sewage (39), surface waters from agricultural watersheds (40). Same as male-specific DNA coliphages, somatic coliphages are detected in raw municipal wastewater and animal fecal contamination sources (41, 42). Male-specific DNA coliphages and somatic coliphages isolated from our study area could be exposed by various fecal contamination sources.

Many studies have been focused on male-specific RNA coliphages because RNA coliphages could differentiate human and nonhuman fecal sources (43). However, if male-specific RNA coliphages were not shown or low level of RNA coliphages were detected, the study may be difficult to interpret the origin of fecal sources. From our results, only male-specific DNA coliphages were

detected in all sampling periods. In surface water samples, both male-specific DNA coliphages and somatic coliphages were highly detected in September (Fig. 3a and 3b) and the highest average temperature on the sampling day in September was 25.2°C (Table 5). This result coincided with the previous study that male-specific DNA coliphages are dominant during the warm seasons whereas male-specific RNA coliphages are more sensitive to warmer temperatures ranging from 25°C to 40°C (44). Compared to warm season, male-specific DNA coliphages were hardly detected in surface water ( $< 5.00 \times 10^0$  PFU/100 mL) in cold season. Due to low detection in cold season, male-specific RNA coliphages might not be detected in this study. In addition, previous study showed similar results that only male-specific DNA coliphages were detected during sampling period (45). It was reported that male-specific RNA coliphages existed with low concentration in coastal waters (46, 47), and suggested that male-specific RNA coliphages might have more weaker lytic tail to replicate to kill the host cells than male-specific DNA coliphages(45). These two reasons could explain that our result showed no recovery of male-specific RNA coliphages from water samples in this study.

Our data showed that both coliphages were more positively correlated with noroviruses than coliforms (Table 6). Several studies

have argued that male-specific RNA coliphages are useful indicator for microbial source tracking because male-specific RNA coliphages could differentiate the contamination sources in environmental resources (48, 49). Although only male-specific DNA coliphages were observed in our study, correlation coefficients were higher than the previous researches (4, 50). The higher correlation between noroviruses and coliphages than coliforms might be explained by site-specific occurrence than seasonality because the result of coliphages seasonality was not corresponded to winter prevalence noroviruses (Fig. 7). Coliphages were also more accurately indicating the presence of noroviruses than bacterial indicators (Fig. 6). This is because coliphages have similar features to enteric viruses such as morphology, size, structure, physiology and survivability (4).

Several environmental parameters were positively or negatively correlated with the levels of coliphages in surface water (Fig. 5). Negative correlation between somatic coliphages average wind speed in surface water samples indicates that the distribution of somatic coliphages may be affected by wind speed, so weak or no wind speed could make coliphages longer stay in water. The level of somatic coliphages were higher in September and wind speed was lower in September (2.7 m/s) than other months (May 4.3 m/s or January 4.8

m/s; Table 5). In addition, the positive relationship between somatic coliphages and relative humidity in surface water could be explained by the nature of viruses structure. The phiX174 (somatic coliphage), which have only capsid proteins with no lipid envelop, was more resistant at 80% relative humidity when viral particles were aerosolized (51). Noroviruses were also detected in high percentage of humidity, and this is because humidity mainly acts on capsid and which might protect genomic materials from virus inactivation (52). The average humidity is higher in September (68.8%) than January (55.5%; Table 5). In addition, somatic coliphages were positively correlated with prep-14 in surface water samples. Rainfall event is well known environmental factor for transporting fecal contamination sources to water environments (6). The storm water might carry somatic coliphages with better survival than male-specific coliphages to surface waters, particularly in slightly higher degree because male-specific coliphages were sensitive to warmer condition than somatic coliphages (4, 18). However, even though average temperature was high (21.1°C) on the sampling day in September (Table 5), the male-specific coliphages were higher than somatic coliphages at this time (Fig. 3a and 3b). Previous study suggested that the ratio of somatic coliphages to male-specific coliphages were low, the fecal contamination sources from older sources whereas at low ratio of somatic coliphages to male-

specific coliphages, fecal sources might be from the recent fecal contamination sources (4). Thus, male-specific coliphages might have been transported to surface water from the recent fecal sources by rainfall event before the sampling day. Water temperature was also positively correlated with somatic coliphages. It could be explained that somatic coliphages were stable than male-specific RNA coliphages between 8°C and 22°C (53), which is similar to our result that average temperature ranged from 8.1°C to 25.2°C during the study period (Table 5).

From seawater samples, rainfall events and male-specific coliphages were highly correlated. (Fig. 6). Prep-0 and prep-14 showed positive correlation with male-specific coliphages ( $r^2 = 0.719$  and  $0.608$ , respectively;  $P < 0.001$ ). It corresponded to the total precipitation data that the prep-0 recorded the highest rainfall in July period (3.1 mm; Table 5), also the prep-14 showed the highest accumulation rainfall data in July period (141.7 mm). The level of coliphages in seawater samples could be influenced by the rainfall at sampling period and the prolonged period of rainfall. From our study, the male-specific coliphages were only detected in July samples in seawater even though the male-specific coliphages were the highest in September in surface water. Because of rainfall event in July, the coliphages might not be

highly detected from surface water samples.

Several environmental parameters such as pH or salinity were correlated with coliphages levels in surface and seawater c results. According to previous research, bacteriophages could survive in unfavorable condition such as pH and salinity that phages could be highly diverse in their families (54).

Because the male-specific DNA and somatic coliphages were could be arouse from various fecal samples, land use analysis were proceeded. Previous study reported that the levels of fecal indicator were higher in commercial, residential and pasture than forest lands (55). In addition, fecal waste fertilizer for agriculture can contaminate the nearby watercourses (56). So fecal wastes could be aroused from various ways. Our data showed that low concentration of coliphages were detected from the Stream 1 sampling sites, which are located around the agriculture areas, whereas the highest concentration of coliphages were recovered from Stream 2, which is located near the residential areas (Fig. 1 and Fig. 4). This may indicate that the site was influenced by point and/or nonpoint sources from the concentrated residential areas. Random fecal sources, such as leaks or illegal

discharges of wastes may also contribute to the water environments (57).

In conclusion, our study area was broadly and seasonally contaminated with male-specific and somatic coliphages in surface and seawater. These findings imply the presence of human viral contamination might be in the study area. One of the hot spot stream in surface water showed the highest levels of both coliphages. In addition, out of many seawater sites, the one that was close to the hot spot stream resulted in the highest amount of male-specific coliphages. This indicates that fecal contamination in surface water may contribute to that in seawater. Several environmental parameters such as rainfall and temperature were correlated with male-specific and somatic coliphages levels in surface and seawater. These factors could affect the levels of coliphages in the water environments. Although male-specific DNA and somatic coliphages could not differentiate the original fecal sources, the hot spot of fecal contamination might be caused by anthropic pollution rather than animal sources based on GIS analysis. From the results of correlation between the noroviruses and indicators, the coliphages, especially the male-specific DNA coliphages, would be useful indicator of noroviruses. Because male-specific DNA coliphage

site-specific occurrence is similar to noroviruses occurrence. Understanding the complex relationships of coliphages patterns could prevent public health risks from viral contamination as demonstrated by analyzing the multi-level integrated coliphage data in a shellfish growing area.

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국문초록

수 환경 내 미생물 마커를 통한  
바이러스 오염도 평가

2017년 2월

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전세계적으로 노로바이러스에 오염된 정착성 수산물 섭취로 인한 질병 발생이 지속적으로 보고되고 있다. 특히, 정착성 수산물 생산지의 상류지역은 노로바이러스와 같은 병원성 미생물을 포함한 분변오염의 근원지로 보여진다. 이와 같은 미생물학적 수질을 모니터링 하기 위해 박테리오파지는 바이러스 오염의 지표미생물로서 사용되어져 왔다. 본 연구는 대한민국 남해 정착성 수산물 생산지역에서 패류 섭취와 관련된 공중 보건 위험을 예방하기 위해 4개의 연구 목적을 가지고 박테리오파지를 통한 수질을 평가하고자 한다: 1. 박테리오파지 분포를 확인, 2. 박테리오파지와 환경인자와의 관계, 3. 노로바이러스와 지표미생물과의 관계, 4 GIS 기반으로 한 주변 지형 조사를 연구 목적으로 진행하였다. 박테리오파지는 수시료에서 분리, 농축, 정제되었다. 총 6번의 시료 채취 결과, 육상수에서 male-specific 파지는  $1.34 \times 10^1$  PFU/L, somatic 파지는  $2.93 \times 10^0$  PFU/L 로 나타났지만 해수에서는 드물게 검출되었고, 육상수에서 두 종류 파지는 9월에 가장 높게 측정이 되었다. 노로바이러스와 관련성이 가장 크게 나타난 지표미생물은 male-specific 파지로 나타났다 ( $r^2 = 0.418$ ;  $P < 0.01$ ). GIS 분석을 이용하여 시료 채취 주변 지형을 조사한 결과, 가장 오염도가 높게 나타난 장소 근처는 거주지역이 밀집되어 있는 것으로 나타났다. 또한 박테리오파지와 환경인자의 상관관계는 습도, 강수량, 온도 등이 양의 상관관계로 나타났다 ( $P < 0.05$ ). 이 연구는 패류 섭취와 관련하여 공중 보건 위험을 최소화 하기 위해 박테리오파지를 포괄적으로 이해함과 동시에 노로바이러스 행동을 예측하는데 있어서 중요한 의미를 가진다.

Key words: 정착성 수산물 생산지역, 박테리오파지, GIS, 환경인자, 지표미생물

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